

***Enterospora nucleophila* (Microsporidia) in gilthead sea bream (*Sparus aurata*): Pathological effects and cellular immune response in natural infections**

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Abstract

Enterospora nucleophila is a microsporidian responsible for an emaciative disease in gilthead sea bream (*Sparus aurata*). Its intranuclear development and the lack of *in vitro* and *in vivo* models hinder its research. This study investigated the associated lesions, its detection by qPCR, and the cellular immune response of naturally infected fish. The intensity of infection in the intestine was correlated with stunted growth and reduced body condition. At the beginning of the outbreaks, infection prevalence was highest in intestine and stomach and in subsequent months the prevalence decreased in the intestine

and increased in hematopoietic organs and stomach. In heavy infections, intestinal histologic lesions were enterocyte hypercellularity and proliferation of rodlet cells. Infected enterocytes had *E. nucleophila* spores in the cytoplasm, and a pyknotic nucleus or karyorrhexis or karyolysis. Lymphocytes were present at the base of the mucosa and eosinophilic granule cells were located between the enterocytes. In intestinal submucosa, macrophage aggregates containing spores were surrounded by lymphocytes and granulocytes, with submucosal infiltration of granulocytes. Macrophage aggregates appeared to develop into granulomata with necrotic areas containing parasite remnants. Immunohistochemistry revealed mast cells as the main type of granulocyte involved. Abundant IgM⁺ and IgT⁺ cells were identified by *in situ* hybridization in the submucosa when intracytoplasmic stages were present. This study describes the lesions of *E. nucleophila* in gilthead sea bream, an important aquaculture species.

Key words

Enterocytozoonidae, histopathology, immunoglobulins, granulocytes, growth retardation, qPCR, Fish

Microsporidia are highly specialized obligate intracellular (intracytoplasmic and/or intranuclear) eukaryotes that are closely related to the Fungi.¹⁸ They have a relatively simple development, consisting of two general stages: merogony and sporogony. Meronts multiply inside the infected host cell, eventually forming sporonts and then spores, which are ultimately released from the host and transmit the infection. There are almost 200 genera and over 1,400

described microsporidian species, though the actual diversity is estimated to be much higher. The host range extends from protists and invertebrates (especially insects and other arthropods) to vertebrates, particularly fish and mammals including humans.⁷ Diverse pathological effects of microsporidia have been described in healthy and immunocompromised humans,^{9,19} in companion animals, and especially in livestock such as bees, silkworms, crabs, lobsters and fish.^{5,15,17,24,25,36,44} Over half of the known genera infect aquatic organisms and some are considered emerging pathogens in the food chain.⁴⁰ About 120 species of microsporidia have been described from fish, and several are involved in diseases of wild, ornamental, experimental models, and farmed stocks. Their impact on fish health is well acknowledged, either producing direct mortalities, diminishing catch value, reducing growth rates, increasing the chances of predation by other animals, or impairing the reproductive or the cardiorespiratory capacities.^{21,23,40}

Enterospora nucleophila is an emerging pathogen in gilthead sea bream (*Sparus aurata*) culture in the Mediterranean area. It is an intranuclear microsporidian species belonging to the family Enterocytozoonidae, which includes human and aquatic hosts. Since its first description in Spanish aquaculture facilities,²⁷ it has also been detected in Italian and Greek gilthead sea bream farms, and in different facilities including on-growing net cages and land-based nurseries.^{1,6} Information related to other fish intranuclear microsporidians is scarce. Mortalities have been reported due to *Nucleospora salmonis* in Chinook and Atlantic salmon,^{4,10} to *Desmozoon lepeophtherii* (syn. *Paranucleospora theridion*) in Atlantic salmon,^{26,46} to *Nucleospora cyclopteri* in

the cleaner fish lump sucker (*Cyclopterus lumpus*),¹³ and to an unidentified intranuclear microsporidium in grouper (*Epinephelus* spp.).⁴⁷

Studies on *E. nucleophila* are hindered by its very small size (spores are 1 x 1.5 µm), its intranuclear development, and the lack of *in vitro* and *in vivo* propagation models. Limited information about the pathology has been presented elsewhere.^{1,27} In this work, we investigated the pathological effects of this infection, its location within the host, and the cellular immune response invoked in naturally infected gilthead sea bream from different disease outbreaks in Mediterranean farms.

Materials and Methods

Fish and samplings

All experimental protocols involving fish were approved by the Ethics and Animal Welfare Committee of IATS, CSIC and Generalitat Valenciana (approval number 151-2014). They were carried out in a registered installation facility (code ES120330001055) in accordance with the principles published in the European Animal Directive (2010/63/EU) and Spanish laws (Royal Decree RD53/2013) for the protection of animals used in scientific experiments. All efforts were made to minimize the suffering of the animals.

Three lots of gilthead sea bream (n = 300 for lot 1 and n = 200 each for lots 2 and 3) on-growing in commercial sea cages in the Western Mediterranean and displaying emaciation, were transported alive to the Institute of Aquaculture Torre de la Sal (IATS-CSIC) facilities. Upon arrival, 200, 92 and 20 fish (from lots 1, 2 and 3, respectively) were sacrificed by overexposure to the anaesthetic (MS-222, 0.1g/L; Sigma). After biometry and necropsy,

intestinal samples were taken for molecular diagnosis by quantitative polymerase chain reaction (qPCR), as well as formalin fixation for histopathology. The remaining 100, 108 and 180 fish of lots 1, 2 and 3, respectively, were kept in the open-flow facilities of IATS-CSIC and mortality was recorded for 152, 79 and 296 days (lots 1, 2 and 3, respectively). Lot 3 was also used to study the progression of the infection in different time points for 296 days post-initial detection (dpid). Fish were sampled at 44 (n = 10), 57 (n = 10), 92 (n = 10), 130 (n = 10) and 296 (n = 21) dpid, and their condition factor (calculated as $100 \times \text{body weight} / \text{length}^3$) and specific growth rate (calculated as $100 \times ((\ln \text{ final body weight}) - (\ln \text{ initial body weight})) / \text{days}$) were calculated. From the same fish, intestines were collected for molecular diagnosis by qPCR. At 92, 130 and 296 dpid, additional samples of stomach, gallbladder, head kidney and spleen were collected for molecular diagnosis. During this time, fish were fed commercial pellets (Biomar) and disease signs and mortalities were recorded.

Simultaneously, another lot of 100 clinically healthy gilthead sea bream (no disease signs of any bacterial or parasitic disease) from the same fish farm, and qPCR-negative for *E. nucleophila*, were reared under the same conditions as the infected ones.

Diagnosis of E. nucleophila infection

qPCR: Oligonucleotide probes targeting the SSU rDNA gene of *E. nucleophila* were designed from an alignment of available Microsporidia SSU rDNA genotypes, including 209 Enterocytozoonidae.¹ Unique *E. nucleophila*

segments, or segments with sufficient variation with respect to the closest taxa, were visually identified in the alignment. Choice of the best probes targeting these segments, according to specificity and thermodynamic properties, was aided by the software package OLIGO 7.³² Two oligonucleotides were chosen as primers for a SYBR qPCR test: forward ENU-101F23: 5' TGC GCT AAT TTG CTT TAT ACG AG 3' and reverse ENU-151R22 5' ATT GCA CCT TAT TCT ACA CTC T3'. PCR reactions were assembled in 20µl volume with 2µl template DNA and 300nM each primer in a SYBR commercial master-mix (PyroTaq Evagreen, CMB, Spain). The reactions were programmed in a thermal cycler with a 15 min Taq activation step (95°C) followed by 40 cycles of denaturation (95°C, 15s); annealing (50°C, 30s) and extension (72°C, 30s). Standards consisted of on 10-fold dilutions with known amounts of a plasmid containing the target gene (covering 7 orders of magnitude) were run with each plate. Only data from reactions with standard curves within an efficiency range ($E = 0.95$ – 1.05), and $R^2 > 0.99$ were accepted. Samples with $C_t < 38$ were considered positive.

DNA was extracted from clinical samples using a robotic system (EpMotion 5070, Eppendorf, Hamburg, Germany) with dedicated DNA extraction kits (Nucleospin, Macherey-Nagel, Germany). The test samples consisted of small pieces of tissue (50-100 mg) or an aliquot (200 µl) of an intestine homogenate. For the homogenates, the tissue (< 2 g) was placed in 5 ml tubes containing ice-cold saline and 3 g of mixed homogenization beads (2 mm, 3.2 mm and 3.5 mm UFO shape), which were then processed in a laboratory blender (Bullet Blender, Next Advance, USA) with 3x 5-min cycles at 4°C. Blood samples were drawn with 0.5 M EDTA-coated syringes. Small

150 aliquots (10 µl) were transferred to microtubes and directly diluted in lysis buffer
151 and processed with a dedicated kit (Nucleospin Blood). Quantification and
152 quality control of extracted DNA samples was monitored with a
153 spectrophotometer (Nanodrop, Thermo Scientific, Spain).

154 Calcofluor-white stain: Paraffin sections (4 µm thickness) were cut and
155 mounted onto electrostatically charged glass slides (Superfrost-Plus; Thermo
156 Fisher Scientific, Spain). Air-dried blood smears were also prepared. They were
157 stained with 0.1% calcofluor white M2R stain in ddH₂O with a drop of 10%
158 potassium hydroxide, for 1 min. The slides were rinsed immediately with 30%
159 ethanol. A light counterstaining was achieved with 0.1% Evans blue solution
160 containing a few drops of glacial acetic acid for 1 min. Slides were dehydrated
161 mounted in DPX and examined using an Olympus BX51 fluorescence
162 microscope under UV excitation light.

163 We use the term “clinically diseased” fish for those in the enzootically
164 affected farms that showed the typical disease signs previously described for *E.*
165 *nucleophila* infection: anorexia, cachexia, swollen abdominal cavity, and
166 transparent intestinal walls)²⁷. Those fish were generally positive by qPCR and
167 histology. On the contrary, “non-clinically diseased” fish were those that did not
168 show clinical signs but were positive by qPCR, and in most cases negative by
169 histology.

171 *Histology, immunohistochemistry and in situ hybridization*

172 Formalin-fixed tissues were dehydrated in a graded ethanol series, embedded
173 in paraffin or methacrylate resin (Technovit 7100, Kultzer, Wehrheim,
174 Germany), sectioned at 1-µm for resin or 4-µm for paraffin) and stained with

Giemsa. Immunohistochemistry was used to discriminate between mast cells and acidophilic granulocytes, among the eosinophilic granule cells. For such purpose, paraffin sections (4 μ m-thick) were mounted on super-frost plus slides and a rabbit-anti-histamine antibody (Sigma-Aldrich) was used to detect mast cells.¹¹

RNA-*in situ* hybridization (RNA-ISH) was used to detect transcripts of IgM and IgT. It was performed using the ViewRNA ISH Tissue 2-plex Assay kit (Affymetrix), which uses a proprietary DNA signal amplification technology allowing simultaneous detection of two target mRNAs in tissue sections with single copy sensitivity. The probe sets were designed using the sequences of gilthead sea bream IgM (accession numbers: JQ811851 and KX599199) and IgT (KX599200 and KX599201) heavy chains, in regions 20-903 and 2-901, respectively. Variable domains and regions not expressed both in the soluble and the membrane-bound forms were excluded, to allow detection of all transcripts regardless of the form. The ISH procedure was performed with 4 μ m-thick tissue sections following the manufacturer's instructions, using a 10 min heat pre-treatment at 90-95°C and a 30 min protease step at 40°C. After probe hybridization and development with Fast Blue and Fast Red substrates, the slides were counterstained with Gill's hematoxylin 25% for 3 min, washed and mounted in aqueous mounting medium.

Statistics

Statistical analyses were performed with GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA). We searched for covariations between infection status and fish biometrical variables using nonparametric Spearman correlation

analyses. Differences between groups were studied with appropriate tests according to the variable studied, as detailed in the Results section. Average Ct values from qPCR reactions are given as the estimated mean \pm CI_{95%}.

Results

Disease signs

Growth arrest and emaciation. The specific growth rate of a naturally infected stock (100% prevalence at the arrival to the laboratory) was determined to be 0.38 during 296 days, about 20% of that of a healthy stock of gilthead sea bream of equivalent size held under the same diet and conditions for the same period (i.e. 1.9). Using grouped data from all the fish identified as infected in this study, a statistically significant positive correlation between the condition factor and the infection intensity (as determined by the qPCR Ct values from intestinal samples) was found (Fig. 1), indicating an inverse relationship between infection intensity and condition factor. In a subsample of naturally infected fish from lot 3 maintained in the laboratory up to 296 dpid and sampled periodically, a clear decrease in the prevalence and intensity of infection and an improved condition factor with time was observed (Figs. 2, 3).

Mortality. In the three lots, mortality started upon arrival of the fish to the IATS facilities and reached 50% or more in a minimum period of 82 days (Table 1).

Other disease signs. Most fish with high infection levels exhibited cachexia, emaciation and lethargy. Cases of extreme cachexia were clearly visible from a dorsal view (Fig. 4). At necropsy, internal organs appeared pale, and the intestine was thinned and transparent and frequently contained clear or

greenish fluid and white faeces in the terminal portion (Fig. 5). Occasionally, ascites was present.

Location of E. nucleophila in the host

Intestinal samples of a subset of fish (n = 20) from clinically diseased gilthead sea bream were selected at an enzootic farm were tested by qPCR for the *E. nucleophila* SSU rDNA gene. The data showed individual-animal differences in the distribution of *E. nucleophila* along the intestine. In general, the anterior intestine had the highest prevalence (75%), whereas posterior and middle intestine segments had lower prevalences (70% and 65% respectively). Ct values obtained for anterior intestine segments were also, on average, lower, indicating higher intensity of infection (average Ct of 28.37, vs 29.82 for middle intestine, and 30.29 for anterior intestine), and this pattern was present in most fish presenting moderate to high infection levels. However, individual patterns varied widely, and only a whole-intestine homogenate could reveal the true prevalence of the stock (100%).

The presence of blood stages was confirmed by qPCR in 44% of a panel of samples from fish with a positive qPCR test for the intestine (n = 63). However, the average Ct values of blood samples were much higher (36.47 ± 0.49 , vs. 30.16 ± 1.19 at the intestine), and often close to the routine positive cut-off value of 38. Indeed, when taking Ct = 40 as a positive cut-off value, 100% of these blood samples were positive.

E. nucleophila was detected by qPCR in organs other than intestine sampled over a 296 dpid period (n = 41, Lot 3; Figure 6). At the time of initial diagnosis, this stock had 100% prevalence based on qPCR of the intestine. *E.*

nucleophila infection tended to become more prevalent in the stomach and especially in the haemopoietic tissues (spleen and head kidney) later in the infection. In the last sampling point (296 dpid), 100% of the sampled fish (n = 21) were positive in spleen and head kidney and 95% in the stomach. In general, as observed with blood samples, samples from organs other than the intestine had relatively high Ct values. For gall bladder samples only 2/41 samples were positive (Ct = 35.9 and 37.8) during the study period. At the last sampling (296 dpid), the infection intensity of haemopoietic tissues was similar or higher than those of intestine (i.e., head kidney samples mean Ct = 33.69 ± 0.652 ; spleen: Ct = 33.80 ± 0.258 ; stomach: Ct = 34.15 ± 1.03 ; intestine Ct = 34.38 ± 2.06).

In contrast to this increase in the prevalence of infection in stomach and haemopoietic organs, the prevalence in the intestine declined to 50% in the first 92 dpid, and to 38% at the last sampling (Fig. 6). All individuals with detectable infection in non-digestive organs also tested positive somewhere in the digestive tract in the first samplings up to 130 dpid. In contrast, at the last sampling point (296 dpid), detection in non-digestive organs did not always coincide with detection in the digestive tract.

Histopathology

Clinically diseased fish (Figs. 7-25). This category included fish in which *E. nucleophila* was clearly detectable by histology (Giemsa and/or calcofluor white-stained specimens), normally in large amounts and mainly in the intestinal mucosa, besides by qPCR. During the initial outbreak detection, the most obvious histologic lesion in the intestine was an increased number of

enterocytes that had a large nucleus, and increase number of rodlet cells occupying the mucosa and disorganizing the normal appearance of the palisade (Figs. 7, 8). At a later stage, *E. nucleophila* was also present within the cytoplasm of rodlet cells, enterocytes and macrophages, either in the mucosa or the submucosa (Figs. 9, 10, 12). The nucleus of infected cells was pyknotic, or had karyorrhexis or karyolysis (Figs. 29-31). Moreover, there was lymphocyte infiltration at the base of the mucosa and frequent eosinophilic granule cells between enterocytes. This profile was typical of infected fish sampled in the farms in winter. In these fish, host cells harbouring cytoplasmic spores were frequently being shed to the gut lumen, with release of microsporidian spores from them (Fig. 11). Merogonial stages and spores were found in cells within blood vessels (Figs. 13-15).

In the subsequent stage (several months after the initial outbreak), macrophage aggregates containing spores were seldom found in the mucosa, but were frequent in the submucosa, where they were often surrounded by an inner layer of lymphocytes and an outer layer of granulocytes (Figs. 16,17), very rarely encapsulated by fibrocytes, and seldom with melanin deposits. At this point, the submucosa displayed inflammatory cellular infiltration with abundant granulocytes, and granulomata were sometimes present in the intestinal serosa. In a later stage of infection (some months later), macrophage aggregates became larger, full of foamy macrophages with ingested spores, and sometimes harbouring what appeared to be remnants of infected rodlet cells (Fig. 18). These aggregates eventually appeared to develop into granulomata with necrotic areas that were filled with remnants of *E. nucleophila* (Fig. 24). In

parallel, epithelial cells showed cytoplasmic rarefaction (Fig. 19) or necrosis (Figs. 20, 21) and the mucosa contained abundant cellular debris (Fig. 25).

Non-clinically diseased fish (Figs. 26-32). Fish that were positive by qPCR (albeit often with high Ct values) but with no *E. nucleophila* stages detected by conventional histology, nonetheless had histologic lesions in the intestine. As above, the lesions were characterized by increased numbers of epithelial cells that had large nuclei and an abnormal chromatin pattern (Fig. 26). Occasionally, merogonial stages were present within these nuclei (Figs. 29-31). Cells appeared frequently vacuolated or with debris. In some cases, rodlet cells were abundant, especially in the posterior segment of the intestine. As for clinically diseased fish, infiltration of lymphocytes and eosinophilic granule cells and early macrophage aggregates (Figs. 27, 28, 32) were common. All these observations correspond to the late disease course and coincide with fish in which *E. nucleophila* was detected by qPCR in haematopoietic organs, such as spleen and head kidney, in which macrophages with *E. nucleophila* remnants accumulated.

Cellular immune response

Immunohistochemistry showed that mast cells were the main type of granulocyte forming the cellular inflammatory infiltrate in the submucosa and mucosa. Very few acidophilic granulocytes were present, even in severe inflammatory infiltrates (Figs. 33-34). RNA-ISH labelled IgM and IgT transcripts in cells in the submucosa when intracytoplasmic stages of *E. nucleophila* were present, and the labelled cells were sometimes in close proximity to the spores. IgT-expressing cells were particularly abundant in the anterior intestine and

even infiltrated in the mucosa in contact with infected cells. IgT- and IgM-expressing cells were also abundant around macrophage aggregates in the submucosa. (Figs. 35-39).

Discussion

Gilthead sea bream with severe *E. nucleophila* infection had arrested growth, emaciation, and thinned intestinal walls. These findings are similar to those recently described in juvenile groupers infected with a related intranuclear enteric microsporidian, including severe emaciation, anorexia, and intestinal oedema with whitish content and a thin transparent wall.⁴⁷ *D. lepeophtherii* infection of Atlantic salmon was previously reported exclusively in the gills and skin, but more recent intestinal infections were associated with ascites, hyperemia and often peritonitis, among other signs.⁴⁶ Whitish faeces are also frequently seen in gilthead sea bream affected by the myxozoan *Enteromyxum leei* or the coccidian *Cryptosporidium molnari*.² Therefore, these signs are not specific for microsporidiosis but common to enteric microparasites affecting feed intake and disrupting intestinal functions such as digestion and absorption of nutrients and minerals and osmotic balance. In the present study, whitish faeces from naturally infected gilthead sea bream were qPCR-positive for *E. nucleophila* (data not shown), suggesting that contamination of the water by faeces is possible.

Stunted growth is a characteristic of this disease.²⁷ In the current study, the body weight of naturally infected fish was approximately half that of the unaffected stock at the farm. Indeed, an abnormally high coefficient of variation in the routine biometrical check-points appears as one of the presumptive

indicators of this infection. In addition, a statistically significant relationship between infection intensity and body condition factor was found in the entire dataset of infected fish sampled during 3 different outbreaks. Stunted growth and poor body condition factor are nonspecific signs and they may also be caused by inadequate diets or feeding regimes, or by other diseases such as enteromyxosis.^{35,37} Growth arrest is common in microsporidian infections, partly due to the induced anorexia. In this study, the specific growth rate in one lot of infected fish was only 1/6 that of normal fish. By comparison, the average weight of moribund grouper infected by an enteric microsporidian was 20-40% of that for uninfected fish, and *D. lepeophtherii*-infected Atlantic salmon weighed only 20–25% of normal growing fish.^{46,47} In rainbow trout experimentally infected with *Loma salmonae*, the onset of significant growth rate suppression was associated with the appearance of gill lesions,³⁸ with xenoma onset, and accompanied by a reduction in feed intake.³⁰ Zebrafish (*Danio rerio*) experimentally infected with *Pseudoloma neurophila* also had a reduced size.³¹ Therefore, the economic losses due to *E. nucleophila* are potentially significant not only due to mortality (which was low to moderate in the sea cages), but more importantly to the severely stunted growth.

In vertebrate and invertebrate hosts, cell-mediated immunity confers protection against microsporidians and the key mechanisms are phagocytosis and encapsulation.^{16,28,42} Most information on fish immune responses against microsporidia is based on xenoma-forming species (reviewed in ³³) and very little is known about intranuclear species. In the current study, the cellular immune response of gilthead sea bream was very mild or absent when *E. nucleophila* was intranuclear in the intestinal mucosa. In this circumstance,

hypercellularity of the mucosa (detected at low magnification) was the main histologic lesion. The cellular immune response apparently started when *E. nucleophila* was located in the cytoplasm of the host cells and/or when it passed into the submucosa. At that point, macrophages and mast cells predominated, as shown by histology and immunohistochemistry, whereas acidophilic granulocytes (equivalent to mammalian neutrophils)⁸ seemed to be depleted, similar to that reported in *E. leei* infections.¹¹ This picture is similar to other fish microsporidia in which the inflammatory response is triggered when xenomas rupture, leading to severe, persistent and extensive cellular proliferation, like in *Loma salmonae* gill infections in salmonids.³ Conversely, in other microsporidians, such as *Spraguea* in the Japanese anglerfish, even heavy infections of the nervous system had no associated lesions.¹² There were high number of IgT-expressing cells in the intestine, particularly the anterior segment, and these cells were distributed across the epithelium and in close proximity to the microsporidia. The abundance of these cells would be in line with the mucosal role of IgT described for teleosts,⁴⁸ and could suggest a role of this immunoglobulin in the local response to *E. nucleophila*.

Foamy macrophages are laden with lipid generated from the massive uptake of modified low-density lipoproteins and the intracytoplasmic accumulation of cholesteryl esters. Foam cells can also be generated as a consequence of infection by persistent pathogens such as *Mycobacterium*, *Chlamydia* and *Toxoplasma* in humans and other mammals.⁴³ These foamy macrophages have been described in other microsporidiosis, such as in AIDS patients with *Encephalitozoon intestinalis* infection in the lamina propria.²² Foamy macrophages containing numerous intracytoplasmic microsporidia were

also observed in granulomatous infiltrates of parrots.¹⁴ In fish, macrophages with foamy cytoplasm have been described ingesting spores of a microsporidian parasite causing liquefaction of the skeletal muscle of the Norway pout, *Trisopterus esmarkii*,²⁹ and in infiltrations in gut, spleen, kidney, heart, liver, gill and dermis of *D. lepeophtherii*-infected Atlantic salmon.⁴⁶ The foamy macrophages that harbour microsporidial stages seem resistant to degradation and it is thought that when the immune system is unable to eliminate substances perceived as foreign, it produces a granuloma encapsulated by fibroblasts, attempting to wall off the non-self-material. The granuloma is therefore typical of fish in a late stage of this disease. The role of macrophages in resistance and clearing of fish microsporidian spores has been described, but some parasites are capable of blocking the intracellular killing mechanisms of phagocytes,³³ thus avoiding destruction by immune cells. In fact, it has been suggested that *P. theridion*-infected macrophages could be responsible for propagating the parasite within the host.⁴¹ The current study confirms the dual role of macrophages. On one hand, the observation of different viable parasite stages within macrophages in the intestinal mucosa and submucosa, and in blood vessels, suggests these cells may act as “Trojan horses”. On the other hand, as the observation of parasite debris within macrophage aggregates suggest that they may combat the parasite in the last stages of the infection, perhaps acting in antigen presentation and attracting other leukocytes. This latter role is also in line with the accumulation of macrophages as well as higher detection of the parasite by qPCR in spleen and head kidney during the late stage of infection. The survival of microsporidians within macrophages has already been described for other species, as they have evolved mechanisms

that allow them to resist destruction within macrophages. For example, phagolysosome formation is prevented by *Glugea hertwigi*,⁴⁵ and *Enterocytozoon cuniculi* is capable of surviving despite the host's active immune response. Latent asymptomatic infections may persist as long as multiplication of the microsporidian and the immune response are in equilibrium.⁴² In the subset of fish from a naturally-infected stock sampled periodically along 296 days in this study, a progressive partial recovery from the infection was noted. Although the data are likely biased by the mortality observed in the stock, a clear decrease in the prevalence from the 100% initial value was still obvious. In addition, infected fish in this stock presented, on average, a much lower intensity and a recovered body condition factor by the end of the follow-up period. This notwithstanding, the presence of fish harbouring the parasite, even if in small numbers, 10 months after an outbreak, it is remarkable and can have obvious epidemiological implications. It is currently unknown if these patterns correspond to a full –recovery stage or to cover infections from which the infection could relapse.¹

Besides the severe disease signs, *E. nucleophila* can cause extensive histopathological changes in acute cases, even when present at lower levels (only detectable by qPCR) in fish that do not show disease signs. Similarly, PCR showed the presence of *N. cyclopteri* in most organs of apparently healthy lumpfish.¹³ The existence of non-symptomatic carriers has relevant consequences for the spread of the disease, and early detection of *E. nucleophila* in non-diseased fish may be useful to prevent further dissemination of infection within farmed stocks, or to avoid stressful handling of the fish that could trigger clinical disease.

E. nucleophila was detected in blood, but its pathological significance is difficult to ascertain. Blood samples were PCR-positive for *E. nucleophila* in only 44% of clinically infected fish from the farms. This finding suggests hematogenous dissemination of *E. nucleophila* within the host. This opens the possibility of cryptic or dormant *E. nucleophila* life stages in other organs as described for *Loma salmonae*.^{34,39} However, in-depth examination of diseased fish including ISH was not conclusive.¹ Experimental infections and time-lapse sequential samplings may be needed to properly determine the presence of *E. nucleophila* life stages in blood and extraintestinal organs.

Several members of the Enterocytozoonidae family have a remarkable impact on aquatic hosts. Pre-stocked ponds of cultured prawns in Thailand had a high prevalence of *Enterocytozoon hepatopenaei*, with the resultant economic losses due to slowed growth and early harvest estimated at \$32,000 per hectare in every culture cycle.²⁰ There are no economic estimates on the impact of *E. nucleophila* in the culture of gilthead sea bream.

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Declaration of Conflicting Interests

The authors declared no competing interest with respect to the research, authorship, and/or publication of this article.

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References

1. Ahmed NH, Caffara M, Sitjà-Bobadilla A, et al. Detection of the intranuclear microsporidian *Enterospora nucleophila* in gilthead sea bream by *in situ* hybridization. *J Fish Dis*. 2019;**42**:809-815.
2. Álvarez-Pellitero P, Sitjà-Bobadilla A. *Cryptosporidium molnari* n. sp. (Apicomplexa: Cryptosporidiidae) infecting two marine fish species, *Sparus aurata* L. and *Dicentrarchus labrax* L. *Int. J. Parasitol*. 2002;**32**:1007-1021.
3. Becker JA, Speare DJ. Transmission of the microsporidian gill parasite, *Loma salmonae*. *Anim. Health Res. Rev.* 2007;**8**:59-68.
4. Bravo S, *Enterocytozoon salmonis* in Chile. *Am. Fish. Soc. (FHS Section Newsletter)* 1996;**24**:12-13.

- 499 5. Brown MJF. Microsporidia: An Emerging Threat to Bumblebees? *Trends.*
500 *Parasitol.* 2017;**33**:754-762.
- 501 6. Caffara M, Sirri R, Mandrioli L, et al. Sindrome emaciata da
502 *Enterospora nucleophila* (Microsporidia) in orate (*Sparus aurata*) allevate
503 in Italia. *Proceedings of the XX Convegno Nazionale SIPI.* Pisa, Italy.
504 2014:44.
- 505 7. Cali A, Takvorian PM. Developmental Morphology and Life Cycles of the
506 Microsporidia. In: Weiss LM, Becnel JJ, ed. *Microsporidia: Pathogens of*
507 *Opportunity.* 1st ed. Ames, Iowa: Wiley-Blackwell; 2014:71-133.
- 508 8. Chaves-Pozo E, Pelegrín P, García-Castillo J. Acidophilic granulocytes
509 of the marine fish gilthead seabream (*Sparus aurata* L.) produce
510 interleukin-1 β following infection with *Vibrio anguillarum*. *Cell Tissue Res.*
511 2004;**316**:189-195.
- 512 9. Didier ES, Weiss LM. Microsporidiosis: not just in AIDS patients. *Curr.*
513 *Opin. Infect. Dis.* 2011;**24**:490-495.
- 514 10. Elston RA, Kent ML, Harrell LH. An intranuclear microsporidium
515 associated with acute anemia in chinook salmon, *Oncorhynchus*
516 *tshawytscha*. *J. Eukaryot. Microbiol.* 1987;**34**:274-277.
- 517 11. Estensoro I, Mulero I, Redondo MJ, et al. Modulation of leukocytic
518 populations of gilthead sea bream (*Sparus aurata*) by the intestinal
519 parasite *Enteromyxum leei* (Myxozoa: Myxosporea). *Parasitology.*
520 2014;**141**:425-440.
- 521 12. Freeman MA, Yokoyama H, Osada A, et al. *Spraguea* (Microsporida:
522 Spraguidae) infections in the nervous system of the Japanese anglerfish,

- 523 *Lophius litulon* (Jordan), with comments on transmission routes and host
 524 pathology. *J. Fish Dis.* 2011;**34**:445-452.
- 525 13.Freeman MA, Kasper JM, Kristmundsson Á,. *Nucleospora cyclopteri* n.
 526 sp., an intranuclear microsporidian infecting wild lumpfish, *Cyclopterus*
 527 *lumpus* L., in Icelandic waters. *Parasit. Vectors.* 2013;**6**:49.
- 528 14.Gelis S, Raidal SR. Microsporidiosis in a flock of tricolor parrot finches
 529 (*Erythrura tricolor*). *Vet. Clin. North Am. Exot. Anim. Pract.* 2006;**9**:481-
 530 486.
- 531 15.Genersch E. Honey bee pathology: current threats to honey bees and
 532 beekeeping. *Appl. Microbiol. Biotechnol.* 2010;**87**:87-97.
- 533 16.Ghosh K, Weiss LM. T cell response and persistence of the
 534 microsporidia. *FEMS Microbiol. Rev.* 2012;**36**:748-760.
- 535 17.Gupta SK, Hossain Z, Nanu MM. et al. Impact of microsporidian infection
 536 on growth and development of silkworm *Bombyx mori* L. (Lepidoptera:
 537 Bombycidae). *Agricult. Nat. Res.* 2016;**50**:388-395.
- 538 18.Han B, Weiss LM. Microsporidia: Obligate intracellular pathogens within
 539 the fungal kingdom. *Microbiol. Spectr.* 2017;**5**:10.
- 540 19.Hassan N, Lim YAL, Mahmud R, et al. Microsporidia infection among
 541 various groups of the immunocompromised patients. *Trop. Biomed.*
 542 2018;**35**:521-530.
- 543 20.Herrero A. Microsporidians: a macro problem in aquaculture. 2017. The
 544 Fish Site ([https://thefishsite.com/articles/microsporidians-a-macro-](https://thefishsite.com/articles/microsporidians-a-macro-problem-in-aquaculture)
 545 [problem-in-aquaculture](https://thefishsite.com/articles/microsporidians-a-macro-problem-in-aquaculture)), 5m Publishing (accessed November 2019).

21. Kent ML, Shaw RW, Sanders JL. Microsporidia in Fish. In: Weiss LM, Becnel JJ, ed. *Microsporidia: Pathogens of Opportunity*. 1st ed. Ames, Iowa: Wiley-Blackwell; 2014:493-520.
22. Liberman E, Yen TS. Foamy macrophages in acquired immunodeficiency syndrome cholangiopathy with *Encephalitozoon intestinalis*. *Arch. Pathol. Lab. Med.* 1997;**121**:985-988.
23. Lom J. A catalogue of described genera and species of microsporidians parasitic in fish. *Syst. Parasitol.* 2002;**53**:81-99.
24. Lom J, Dyková I. Microsporidian xenomas in fish seen in wider perspective. *Folia Parasitol.* 2005;**52**:69-81.
25. Morado JF. Protistan diseases of commercially important crabs: A review. *J. Invertebr. Pathol.* 2011;**106**:27-53.
26. Nylund S, Nylund A, Watanabe K, et al. *Paranucleospora theridion* n. gen., n. sp. (Microsporidia, Enterocytozoonidae) with a life cycle in the salmon louse (*Lepeophtheirus salmonis*, Copepoda) and Atlantic salmon (*Salmo salar*). *J. Eukaryot. Microbiol.* 2010;**57**:95-114.
27. Palenzuela O, Redondo MJ, Cali A, et al. A new intranuclear microsporidium, *Enterospora nucleophila* n. sp., causing emaciative syndrome in a piscine host (*Sparus aurata* L.), prompts the redescription of the family Enterocytozoonidae. *Int. J. Parasitol.* 2014;**44**:189-203.
28. Pan G, Bao J, Ma Z, et al. Invertebrate host responses to microsporidia infections. *Dev. Comp. Immunol.* 2018;**83**:104-113.

29. Pulsford A, Matthews RA. Macrophages and giant cells associated with a microsporidian parasite causing liquefaction of the skeletal muscle of the Norway pout, *Trisopterus esmarkii* (Nilsson). *J. Fish Dis.* 1991;**14**:67-78.
30. Ramsay JM, Speare DJ, Daley J. Timing of changes in growth rate, feed intake and feed conversion in rainbow trout, *Oncorhynchus mykiss* (Walbaum), experimentally infected with *Loma salmonae* (Microspora). *J. Fish Dis.* 2004;**27**:425-429.
31. Ramsay JM, Watral, V, Schreck CB, et al. *Pseudoloma neurophilia* infections in zebrafish *Danio rerio*: effects of stress on survival, growth, and reproduction. *Dis. Aquat. Organ.* 2009;**88**:69–84.
32. Rychlik W. OLIGO 7 primer analysis software. *Methods Mol. Biol.* 2007;**402**:35–60.
33. Rodriguez-Tovar LE, Speare DJ, Markham RJF. Fish microsporidia: Immune response, immunomodulation and vaccination. *Fish Shellfish Immunol.* 2011;**30**:999-1006.
34. Sánchez JG, Speare DJ, Markham RJF. Normal and aberrant tissue distribution of *Loma salmonae* (Microspora) within rainbow trout, *Oncorhynchus mykiss* (Walbaum), following experimental infection at water temperatures within and outside of the xenoma-expression temperature boundaries. *J. Fish Dis.* 2000;**23**:235-242.
35. Sekiya M, Setsuda A, Sato H, et al. *Enteromyxum leei* (Myxosporaea: Bivalvulida) as the cause of myxosporean emaciation disease of farmed olive flounders (*Paralichthys olivaceus*) and a turbot (*Scophthalmus maximus*) on Jeju Island, Korea. *Parasitol. Res.* 2016;**115**:4229-4237.

36. Shields JD. Diseases of spiny lobsters: A review. *J. Invertebr. Pathol.* 2011;**106**:79-91.
37. Sitjà-Bobadilla A, Palenzuela O. Enteromyxum Species. In: Woo PTK, Buchmann K, ed. *Fish Parasites: Pathology and Protection*. 1st ed. Oxfordshire, UK: CAB International; 2012:163-176.
38. Speare, DJ, Daley J, Markham RJF, et al. *Loma salmonae* associated growth suppression in rainbow trout (*Oncorhynchus mykiss*) occurs during early onset xenoma dissolution as determined by in situ hybridization and immunohistochemistry. *J. Fish Dis.* 1998;**21**:345-354.
39. Speare DJ, Beaman HJ, Daley J. Effect of water temperature manipulation on a thermal unit predictive model for *Loma salmonae*. *J. Fish Dis.* 1999;**22**:277-283.
40. Stentiford GD, Becnel JJ, Weiss LM, et al. Microsporidia—Emergent Pathogens in the Global Food Chain. *Trends Parasitol.* 2016;**32**:336-348.
41. Sveen S, Øverland H, Karlsbakk E, et al. *Paranucleospora theridion* (Microsporidia) infection dynamics in farmed Atlantic salmon *Salmo salar* put to sea in spring and autumn. *Dis. Aquat. Org.* 2012;**101**:43-49.
42. Valencakova A, Halanova M. Immune response to *Encephalitozoon* infection review. *Comp. Immunol. Microbiol.* 2012;**35**:1-7.
43. Valledor AF, Lloberas J, Celada A. Macrophage Foam Cells. In: John Wiley & Sons Ltd, ed. *eLS*, 1st ed. Chichester, UK: 2015;1-10.
44. Vergneau-Grosset C, Larrat S. Microsporidiosis in Vertebrate Companion Exotic Animals. *J. Fungi (Basel)*. 2015;**2**:3.

- 616 45. Weidner E, Sibley LD. Phagocytized intracellular microsporidian blocks
617 phagosome acidification and phagosome-lysosome fusion. *J. Protozool.*
618 1985;**32**:311-317.
- 619 46. Weli SC, Dale OB, Hansen H, et al. A case study of *Desmozoon*
620 *lepeophtherii* infection in farmed Atlantic salmon associated with gill
621 disease, peritonitis, intestinal infection, stunted growth, and increased
622 mortality. *Parasit. Vectors* 2017;**10**:370-383.
- 623 47. Xu LW, Liu XH, Zhang JY, et al. Outbreak of enteric microsporidiosis of
624 hatchery-bred juvenile groupers, *Epinephelus* spp., associated with a
625 new intranuclear microsporidian in China. *J. Fish Dis.* 2017;**40**:183-189.
- 626 48. Zhang YA, Salinas I, Li J, et al. IgT, a primitive immunoglobulin class
627 specialized in mucosal immunity. *Nat. Immunol.* 2010;**11**:827-835.

Legends to figures

Figure 1. Correlation between intensity of infection by *Enterospora nucleophila* (qPCR Ct values) and fish body condition factor (CF). Higer intensity of infection (lower Ct) correlates with worse CF in fish obtained from different outbreaks (Lot 1: n = 61, Lot 2: n = 92 and Lot 3: n = 48). Each dot represents the data from a single infected fish. $CI_{95\%} = [0.1236, 0.3893]$. r_s = Spearman correlation coefficient, $P < 0.05$.

Figures 2-3. *Enterospora nucleophila*-infected fish from a stock with 100% prevalence and high intensity (Lot 3) were maintained in the laboratory up to 296 days post-initial detection (dpid) and sampled periodically. **Figure 2.** Correlation between intensity of infection (qPCR Ct values) and fish body condition factor (CF) in samples grouped by sampling period in days after initial infection (dpid), represented as different shape-coded symbols (n = 28). $CI_{95\%} = [0.2673, 0.7713]$. In spite of the mortality bias, the data illustrates a recovery trend in surviving fish. **Figure 3.** Prevalence of infection decreases with time along the study. Each dot represents the infection prevalence (based on qPCR) at a given sampling point. The dotted line represents the trend across time. $CI_{95\%} = [-0.999, 1.086]$. r_s = Spearman correlation coefficient, $P < 0.05$.

Figures 4-5. *Enterosporora nucleophila* infection, gilthead sea bream. **Figure 4.** An infected fish with extreme cachexia and anorexia. **Figure 5.** Thinned and transparent intestinal walls (arrow) and white faeces in the terminal portion (arrowheads).

Figure 6. Prevalence of *Enterospora nucleophila* infection determined by qPCR in different organs from naturally infected fish maintained at the laboratory and sampled periodically up to 296 days post-initial detection (dpid) of infection in the lot of fish. Sto = stomach; Int = intestine; G bld = gall bladder; H Kid = head kidney; Spl = spleen.

Figures 7-15. Severe *Enterospora nucleophila* infection, intestines, gilthead sea bream. Resin sections, Giemsa stain. **Figs. 7-8.** The mucosa contains abundant infected rodlet cells that contain intranuclear *E. nucleophila* life stages (arrowheads). **Fig. 9.** Macrophage engulfing an infected rodlet cell (encircled image) in the mucosa. **Fig. 10.** Infected rodlet cells in the mucosa and submucosa (arrows). **Fig. 11.** Detail of mature spores within hypertrophied enterocytes. Note spores shed into the gut lumen (arrowhead). **Fig. 12.** Spores within macrophages in the submucosa (arrows). **Fig. 13.** Blood cells contain initial intranuclear merogonial stages. **Figs. 14 and 15.** Macrophages within blood vessels harbour merogonial stages (Fig 14) and spores (Fig 15).

Figures 16-18. Severe *Enterospora nucleophila* infection, intestines, gilthead sea bream. Resin sections, Giemsa stain. **Fig. 16.** Foamy macrophages containing spores are surrounded by lymphocytes. **Fig. 17.** Eosinophilic granule cells (arrowheads) surrounding a macrophage aggregate. **Fig. 18.** Degenerated foamy macrophages contain spores and remnants of the walls of infected rodlet cells.

Figures 19-25. Advanced *Enterospora nucleophila* infection, intestines, gilthead sea bream. Resin sections, Giemsa stain. **Fig. 19.** Cytoplasmic rarefaction of mucosa and submucosa (arrows). **Fig. 20.** Inflammation in the submucosa (arrow). **Fig. 21.** Detail of macrophages within the submucosa in the arrowed area in Fig. 20. **Fig. 22.** Eosinophilic granule cells within the lamina propria-submucosa. **Fig. 23.** Macrophage aggregate in the serosa (arrow), surrounded by abundant eosinophilic granule cells. **Fig. 24.** Necrotic granuloma in the submucosa. **Fig. 25.** Extensive necrosis in the mucosa. **Figures 26-27.** *Enterospora nucleophila* infection without disease signs, intestine, gilthead sea bream. Resin sections, Giemsa stain. **Fig. 26.** Hypercellularity in the mucosa. **Fig. 27.** Severe inflammatory infiltrate in the submucosa (double-arrowed line).

Figures 28-32. *Enterospora nucleophila* infection without disease signs, intestine, gilthead sea bream. Resin sections, Giemsa stain. **Fig. 28.** Numerous eosinophilic granule cells in the submucosa. **Figs. 29-31.** Nuclei of mucosal cells with anomalous chromatin, some containing parasites (arrows). **Fig. 32.** Macrophage aggregate in the submucosa (arrow).

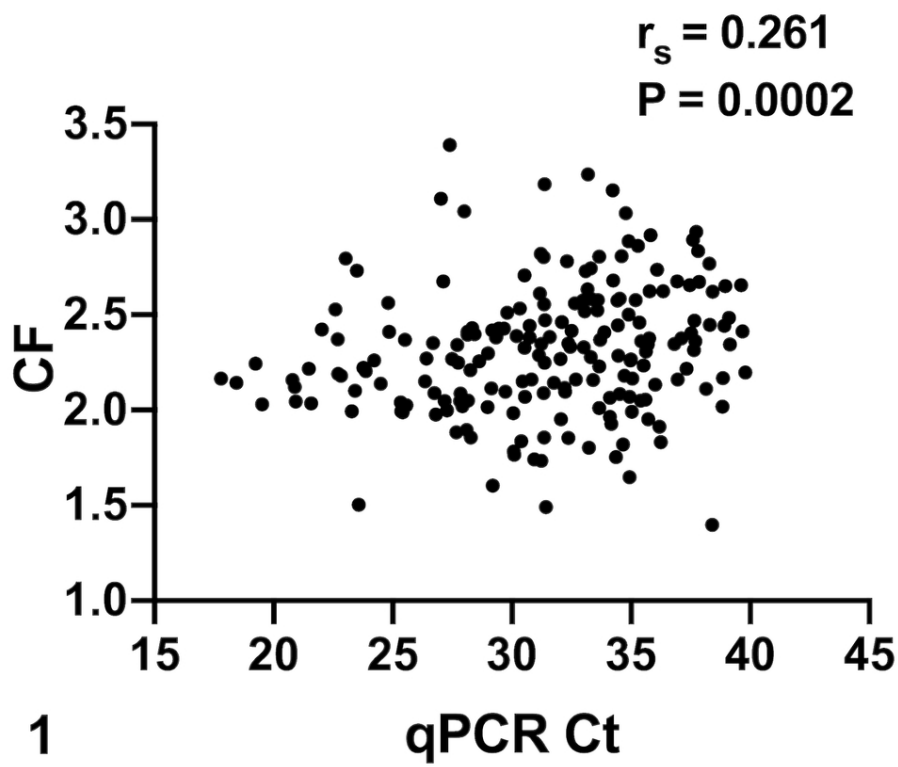
Figures 33-39. Severe *Enterospora nucleophila* infection, intestines, gilthead sea bream. **Figs. 33-34.** Abundant mast cells (arrowheads) in the submucosa. Groups of spores are present (arrows). Immunohistochemistry for histamine (brown labelling). **Figs. 35-39.** Anterior intestinal segment. *In situ* hybridization (ISH) for IgM (blue) and IgT (fuchsia). **Fig. 35.** IgT transcripts within cells in the mucosa. **Fig. 36.** IgM⁺ and IgT⁺ cells are adjacent to each other in the submucosa. **Figs. 37-39.** IgM⁺ cells (black arrowheads) and IgT⁺ cells (red

705 arrowheads) surround parasite spores (arrows). A macrophage aggregate is
706 surrounded by IgM⁺ cells (Fig. 38).

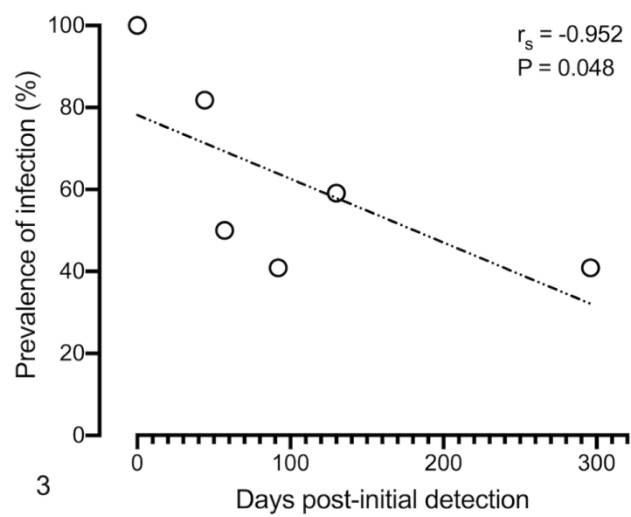
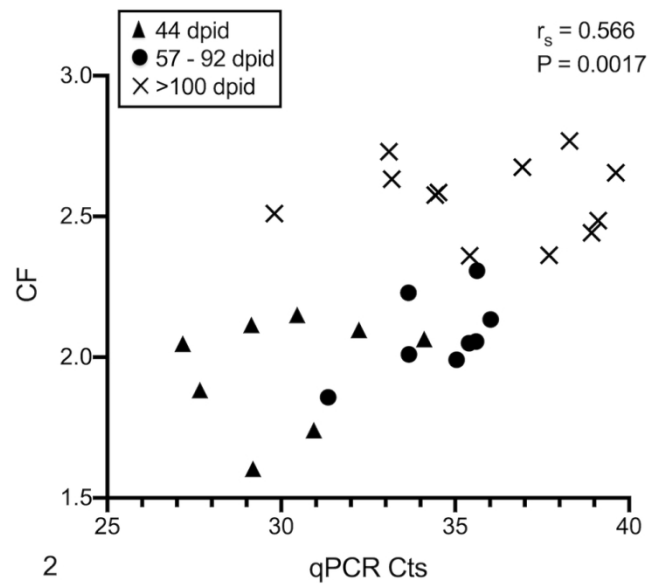
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Table 1. Epidemiology of natural *Enterospora nucleophila* infection in 3 lots (L) of gilthead sea bream. Diagnosis was done by qPCR except in *, by histology.

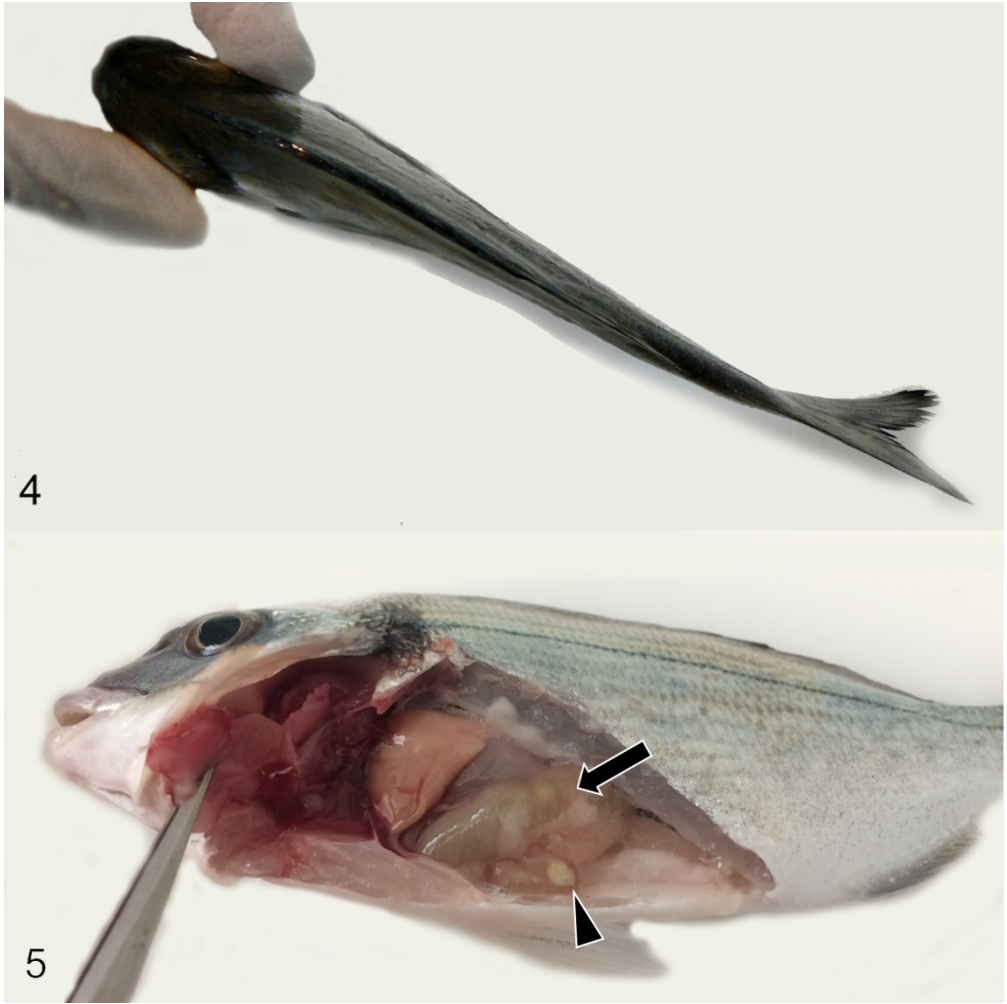
	L-I	L-II	L-III
Prevalence of infection at arrival (%)	30*	100	100
Mortality (%)	100	54.8	50
Period (days)	152	79	82



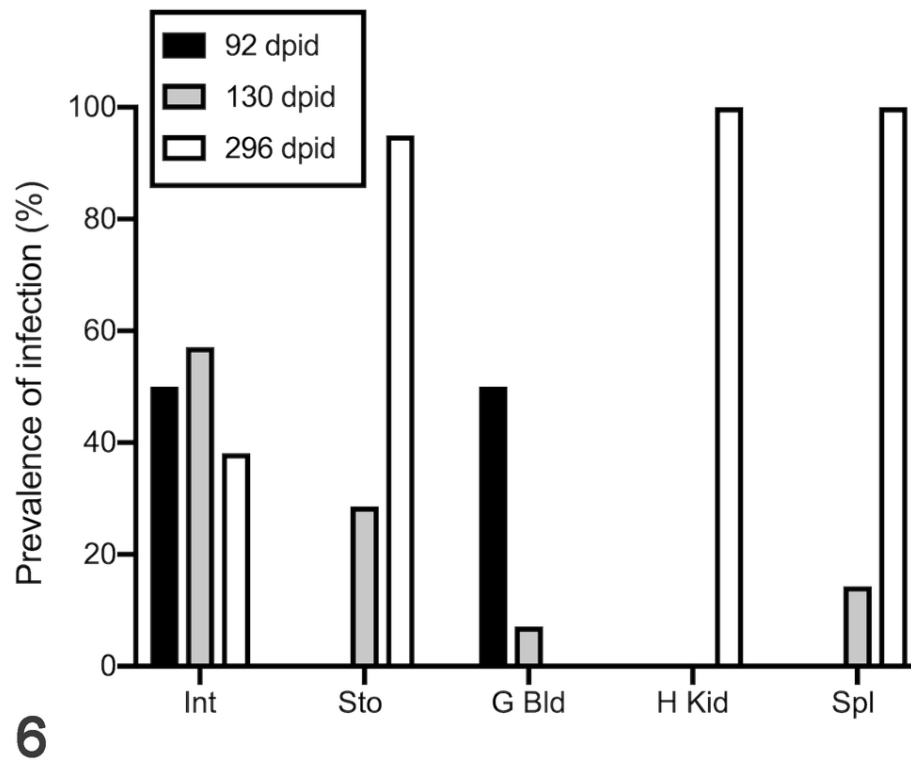
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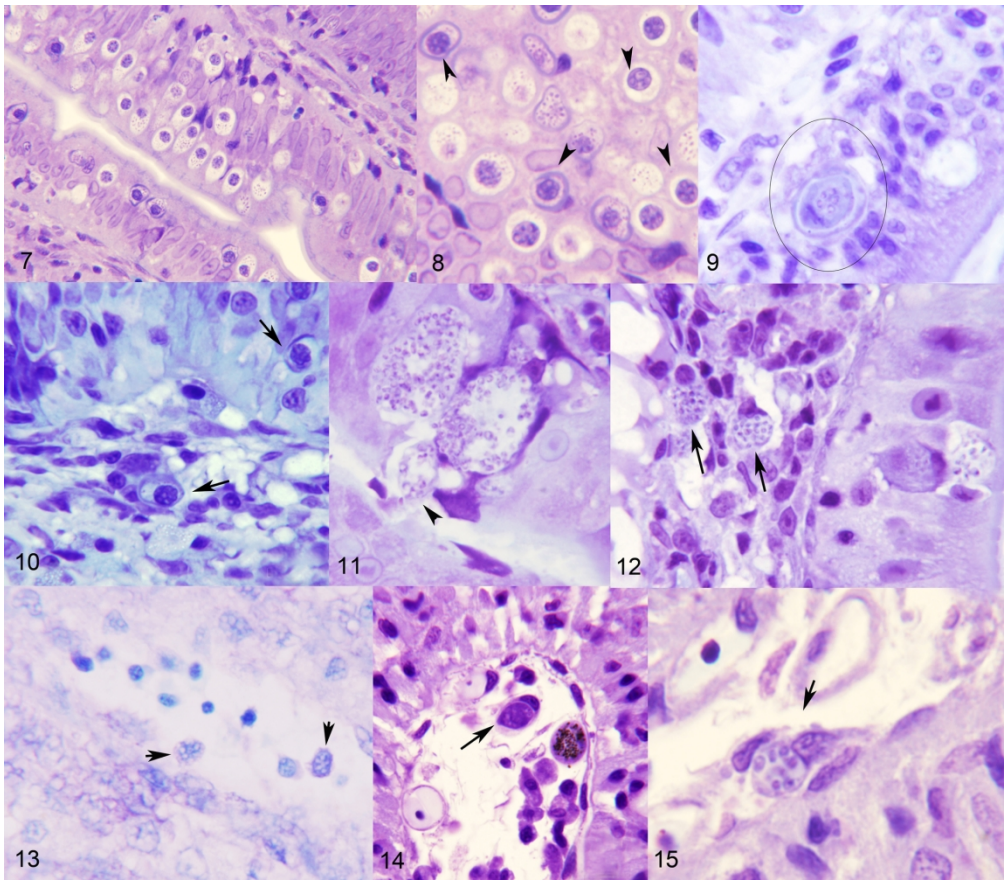
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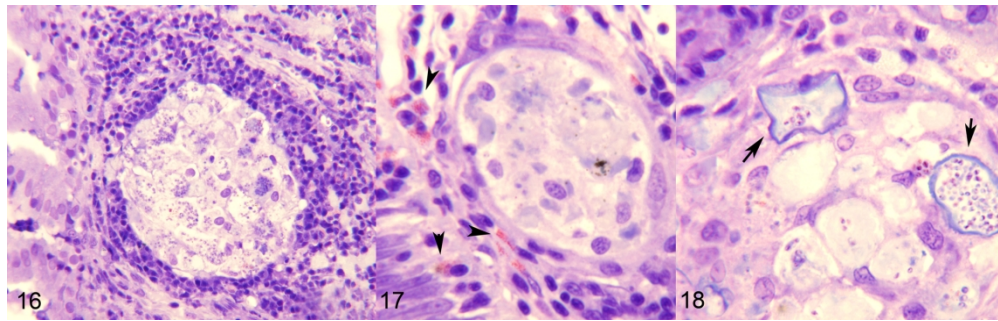
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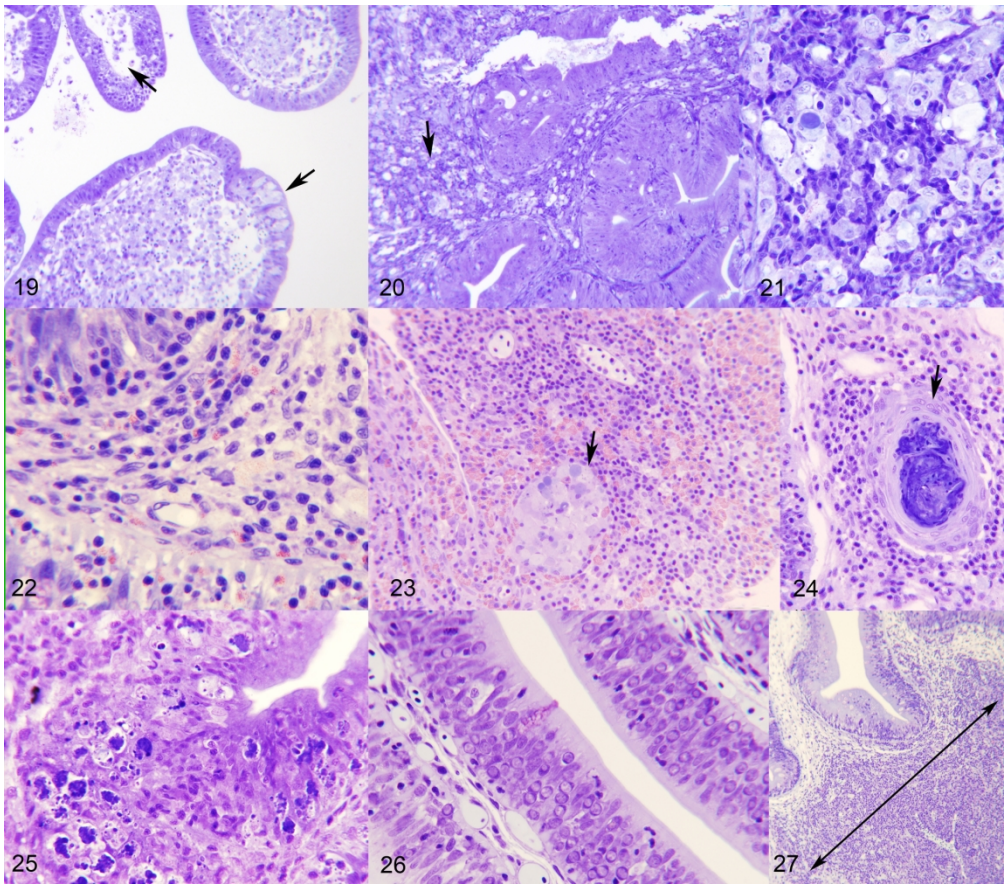
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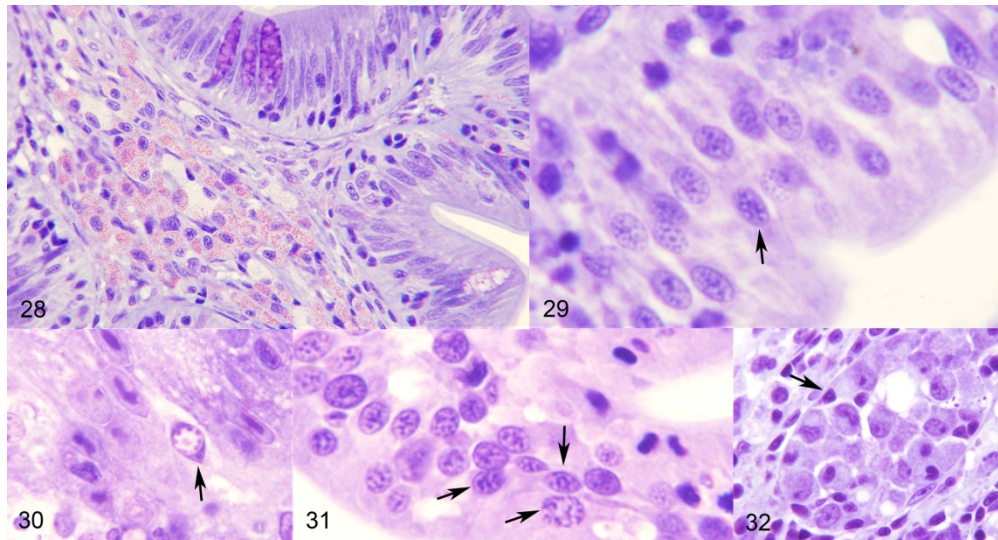
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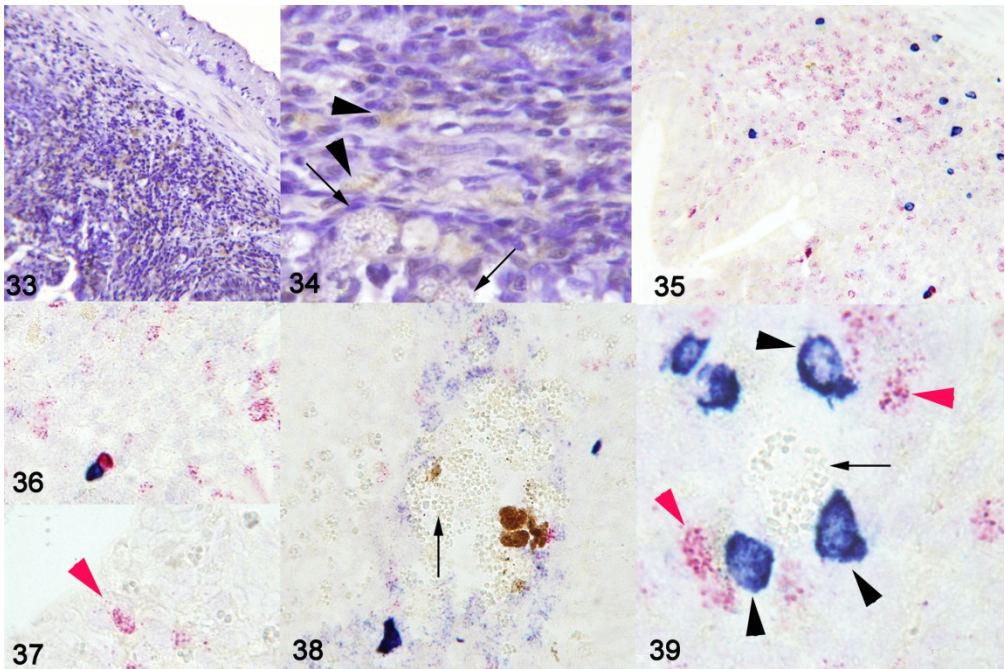
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